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In re Patent Application of	**BOX AF**
Frederic KLEIN et al	Group Art Unit: 1645
Application No.: 09/155,982	Examiner: Virginia Allen Portner
Filed: October 9, 1998	Confirmation No.: 9420
For: MEANS FOR DETECTING BACTERIA OF THE TAYLORELLA EQUIGENITALIS SPECIES AND THEIR BIOLOGICAL APPLICATIONS	

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SUPPLEMENTAL REPLY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Further to our Paper dated April 14, 2003, applicants wish to submit the enclosed copy of the Poster which was discussed in the previously submitted Gradinaru Declaration in paragraphs 20-24:

20. Results relating to this invention were presented in an abstract and poster at the IXth International Symposium of Veterinary Laboratory Diagnosticians, on June 2-5, 1997, at College Station, Texas, USA. The Abstract is attached hereto as Appendix B. As shown therein, the monoclonal antibodies of the invention were used in an indirect immunofluorescence (IIF) test to evaluate their sensitivity and specificity. Several experiments were done.

21. In the first experiment, the claimed monoclonal antibodies detected 4 reference strains and 253 (97,7%) out of 259 field strains, as tested by French Reference Laboratory of Contagious Equine Metritis (CEM).

22. In the second experiment, five French State Veterinary Laboratories compared the results of 1014 routinely CEM swabs by three methods: IIF using the monoclonal antibodies of the invention, IIF using polyclonal antibodies, and culture. In this test, the culture was considered the reference test. Out of 1014 samples, only one *Taylorella equigenitalis* was isolated and identified in culture, but 58 (6%) were positive with the IIF-Mabs kit and 409 (40%) with the polyclonal IIF test.

23. This experiment shows a sensitivity of 97.7% and specificity of 94%, the indirect immunofluorescence test using monoclonal antibodies could be a valuable test for the diagnosis of contagious equine metritis.

24. These experiments show the beneficial and unexpected results achieved by the instant invention. It would not have been known, prior to the instant invention, that such sensitivity and specificity could be achieved.

As discussed in the "Introduction," the kits presently available for detecting *T. equigenitalis* are insufficient. All positive samples must be confirmed by culture isolation due to a lack of specificity and sensitivity. As described under "Materials and Methods," the selected clones were tested by IIF, serum agglutination test, dot-blotting, dot-blotting with denaturation and immunoblotting.

As described in the "Results," four monoclonal antibodies which recognize the epitopes situated in proteins of 150, 120, 52.7 and 22 kDa were used to prepare an IIF-mAbs kit for CEM field diagnostic. Out of 259 field strains, 253 (97.7%) were recognized by the kit. The 6 strains which were negative were not fully characterized, and could actually belong to "*Taylorella*-like" strains.

In the second experiment, out of 1014 samples, only one *Taylorella equigenitalis* was isolated and identified by culture, but 58 (6%) were positive with the kit of the invention and 409 (40%) with the polyclonal IIF test. "This means that for the routine diagnostic, the Pourquier IIF-mAbs kit® increases significantly the specificity up to 94% versus a specificity of 60% with the polyclonal IIF test.

The authors conclude that "[w]ith a sensitivity of 97,7% and a specificity of 94%, the indirect immunofluorescence test using monoclonal antibodies (Pourquier's IIF-mAbs kit®) can be a valuable test for the routine diagnostic of contagious equine metritis."

This Poster, as described in the previously submitted Declaration, evidences the unexpected and beneficial results of the instant invention.

In view of the above, together with the previously submitted Reply, withdrawal of the rejections of record is believed to be in order. Thus, a Notice of Allowance is respectfully requested.

In the event that there are any questions relating to this amendment or the application in general, it would be appreciated if the Examiner would contact the undersigned attorney by telephone at (650) 622-2360 so that prosecution of the application may be expedited.

Respectfully submitted,

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CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this paper is being facsimile transmitted to Examiner Virginia Portner (Fax No. 703-308-4242) at the U.S. Patent and Trademark Office on April 17, 2003.

April 17, 2003
Date

Amy Scipione
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TAYLORELLA EQUIGENITALIS TEST

The diagnostic of contagious equine metritis by indirect immunofluorescence test using monoclonal antibodies.

REF. : P01410

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INTRODUCTION

The laboratory diagnostic of contagious equine metritis (CEM) is based essentially on the isolation of *Taylorella equigenitalis* by bacteriological culture from the swabs and identification of the agent by morphological and biochemical criteria.
Because of the considerable fragility of the bacterium, its slow-growing character and its growth inhibition caused by other contaminating bacteria, bacteriological culture is not always a reliable diagnostic.
The bacterium can be identified by indirect immunofluorescence (IIF) which is a sensitive and rapid method (Vaissaire and Tram, 1992). This test has been introduced in France for the routine diagnostic of CEM from swabs since 1995 but because of the poor specificity, all positive samples must be confirmed by culture isolation. For accurate antigenic detection, there are two essential difficulties : the antigenic cross-reactivity of the agent, which is disputed by some authors, and especially, the heterogenic spectra of the antibody reactivity of the polyclonal antisera, which may contain antibodies of a wide range of bacteria other than T. equigenitalis.
For these reasons, we have developed an immunofluorescence test using monoclonal antibodies (IIF-mAbs).

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MATERIALS AND METHODS

Bacteriological culture

All strains were grown in chocolate agar plates with and without actidione and streptomycin (AES Laboratoire, Combourg, France) and incubated in a 7% CO2 humid atmosphere. The isolated bacteria were tested for catalase, cytochrome oxydase activity and serum agglutination test (SAT), using a polyclonal rabbit antiserum (CNEVA, Alfort, France).

Production and selection of hybridomas

After two fusions, 60 wells were tested in IIF for the detection of hybridomas that produced anti-T. equigenitalis antibodies and 35 (58%) were positive.
Monoclonal antibodies (mAbs) were produced against *Taylorella equigenitalis* using two reference strains : R1-16 (streptomycin sensible) and R2-19 (streptomycin rezistante) obtained from Dr Vaissaire (CNEVA, Alfort, France).

Characterization of monoclonal antibodies :

The 16 selected clones were tested by IIF (Fig. 1), serum agglutination test, dot-blotting, dot-blotting with denaturation (treatment with 2 mM ß-mercaptoethanol and heating at 100°C) and immunoblotting (Fig. 2). The sigma immunotype kit was used for the determination of the mouse monoclonal antibody isotype.

SDS-PAGE

Sample solvent : 0.1 M Tris-HCl pH 6.8 ; 10% glycerol ; 2% SDS ; 2mM ß-mercaptoethanol and 0.01% bromophenol blue. For the separation of bacterial proteins, a discontinuous SDS-PAGE was used : the separating gel was made from 12% acrylamide and the staking gel from 4% acrylamide. The electrophoresis was performed at 100V, 50 mA (constant current) for 10h in a vertical slab gel unit.
For the visualisation of the bands in polyacrylamide matrice, we used Coomassie R350 staining (Pharmacia-Biotech, France) and for the LPS components, silver staining.

RESULTS

Out of the 79 hybridoma clones shown to express antibodies to T. equigenitalis by IIF, 16 were selected and characterised (Table 1).
Four monoclonal antibodies which recognised the epitopes situated in proteins of 150, 120, 52.7 and 22 kDa were further used to prepare an IIF-mAbs kit® (Pourquier Institute, Montpellier, France) for CEM field diagnostic.

This kit was tested in two experiments :

Firstly, by French Reference Laboratory of CEM (CNEVA, Alfort), in order to identify 4 reference strains and 259 field strains (years : 1993-1998) which were isolated by the standard protocol used in the control of CEM in France : immunofluorescence using polyclonal antibodies and/or culture (isolation by bacteriological culture and identification by morphological and biochemical criteria).

Secondly, by five French State Veterinary Laboratories, which compared the results of 1014 routinely CEM swabs by three methods : the Pourquier's IIF-mAbs kit®, the IIF using polyclonal antibodies (CNEVA Alfort) and the culture. In this experiment, the culture was considered the reference test.

Out of 259 field strains, 253 (97,7%) were recognised by the kit. The 6 strains which were negative by IIF-mAbs kit® were not fully characterised, but they could belong to the " Taylorella like " strains.
In the second experiment, out of 1014 samples, only one *Taylorella equigenitalis* was isolated and identificated in culture, but 58 (6%) were positive with the IIF-mAbs kit® and 409 (40%) with the polyclonal IIF test (Table 2). The swab which was positive in culture, was also positive in both immunofluorescence tests.
This means that for the routine diagnostic, the Pourquier IIF-mAbs kit® increases significantly the specificity up to 94% versus a specificity of 60% with the polyclonal IIF test.

CONCLUSIONS

With a sensitivity of 97,7% and a specificity of 94 %, the indirect immunofluorescence test using monoclonal antibodies (Pourquier's IIF-mAbs kit®) can be a valuable test for the routine diagnostic of contagious equine metritis.

Table 1

Characterisation of 16 monoclonal antibodies to the T. equigenitalis R-19 Strain

	mAbs désignation	I.I.F.	SAT	Immunoblot	Dot blot with denaturation	Dot blot Without denaturation	Monoclonal specificity (kDa)	Isotype 1
1	3B6.1	+	+	+	+	+	150	IgM
2	3B6.4	+	+	-	-	+		IgM
3	3B6.11	+	+	-	-	+		IgM
4	7B7.1	+	-	-	-	+		IgG1
5	7B7.7	+	-	+	+	+	34.4+40	IgG1+IgG3
6	7B7.10	+	+	+	+	+	22 (LPS)	IgG1
7	7B8.1	+	+	+	+	+	52.7	IgG3
8	7C4.10	+	+	+	+	+	52.7	IgG3
9	7D7.3	+	+	+	+	+	22 (LPS)	IgM
10	7D7.12	+	-	-	+	+		IgM
11	7D7.16	+	+	-	-	+		IgM
12	10C4.17	+	+	-	-	+		IgG3
13	10C9.6	+	+	-	-	+	120	IgG2b
14	11C9.1	+	+	+	+	+	22 (LPS)	IgG2b
15	11C9.4	+	+	+	+	+	22 (LPS)	IgG2b
16	11c9.5	+	+	+	+	+	22 (LPS)	IgG2b

Table 2

Comparative results between IIF-mAbs, IIF-polyAbs and culture realised by five French State Veterinary Diagnostic Laboratories

Laboratory	Number of samples	Positive samples by I.I.F.-mAbs	Positive samples by I.I.F.-polyAbs	Positive samples in both tests	Culture isolation
LVD 01	74	0 (0%)	15 (20%)	0 (0%)	0
LVD 49	135	3 (2%)	33 (24%)	3 (2%)	1
LVD 53	178	8 (4%)	23 (13%)	6 (3%)	0
LVD 76	145	14 (10%)	61 (42%)	5 (3%)	0
LDO	482	33 (7%)	277 (57%)	29 (6%)	0
Total	1014	58 (6%)	409 (40%)	43 (4%)	1

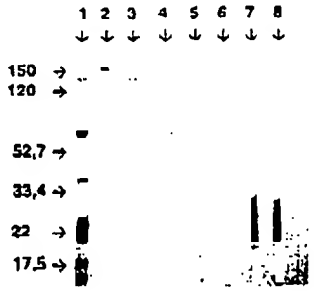
Fig. 1 : Imm . .orescence test (R19 referenc .) using mAbs anti-T. equigenitalis x 100 imersion

Immunofluorescence test

After drying (15 min at 37 °C), the slides were fixed in pure acetone for 15 min at room temperature. The slides were incubated with 30 µl of monoclonal antibodies (30 min at 37°C). The slides were washed in a PBS bath under agitation for 15 min. After rinsing in distilled water and drying, the slides were incubated 30 min at 37°C with 30 µl of solution of fluorescein isothiocyanate conjugate. Finally, the slides were washed in distilled water, dried as above, mounted in glycerin with 1% PBS and examinated with a fluorescence microscope.

Fig. 2. Localisation of specific protein bands recognized by mouse positive serum and by mAbs in immunoblotting.

lane 1 : mouse positive serum
lane 2 : mAb 3B6.1 (150 kDA)
lane 3 : mAb 11C9.1(120 kDA)
lane 4 : mAb 7B8.1 (52.7kDA)
lane 5 : mAb 7B7.7 (33.4+40kDA)
lane 6 : mAb 7C4.10 (52.7 kDA)
lane 7 : mAb 7B7.10 (22 kDA) LPS
lane 8 : mAb 7D7.3 (22 kDA) LPS



Immunoblotting

The protein bands were transferred from the gel onto an immobilon PVDF membrane (Millipore Corp. Saint-Quentin-en-Yvelines, France) by electroblotting using a MiniTrans-Blot electrophoretic transfer cell (Bio Rad) with a transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) at 100 V, 250 mA for 1 h. In order to verify the protein bands on the membranes, we used the Colloidal Gold Total Protein Stain (Bio-Rad). After the transfer, the membranes were submerged in a blocking solution (3 % gelatin in 20 mM Tris and 0.5 M NaCl) and rinsed by gentle agitation in wash solution (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20). The membranes were then exposed to dilutions of mAbs diluted from 1 :100 to 1 :1000 in antibody buffer (20 mM Tris, 0.5M NaCL, 0.05% Tween 20, 1% gelatin) for 180 min at 25°C. The binding of mAbs to the peptide bands was visualised with a goat anti-mouse IgG (H+L) alkaline phosphatase (AP) conjugate (Bio-Rad) (1/2000 dilution) and AP substrate solution (Bio-Rad). A positive serum from mice immunized with T. equigenitalis reference strain and negative serum from a nonimmunized mouse were used to check the procedure.